Supporting Information for:

SNAP-tagged nanobodies enable reversible optical control of a G protein-coupled receptor via a remotely tethered photoswitchable ligand

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Method	Spatial Precision	Genetic Targeting	Temporal Control	Subtype-Specificity	Targets Native Receptors
Opsin-based	Yes	Yes	Yes	N/A	No
DREADDs	No	Yes	No	N/A	No
Photoswitchable Tethered Ligands	Yes	Yes	Yes	Yes	No
Caged & Photochromic Ligands	Yes	No	Yes	Limited by parent pharmacology	Yes
DART	No	Yes	No	Limited by parent pharmacology	Yes
Modulatory Nanobodies	No	No	No	Yes	Yes
Nanobody-Photoswitch Conjugate	Yes	Yes	Yes	Yes	Yes

Table S1. Methods for manipulation GPCRs and other membrane receptors. Over the last decade, many approaches have been developed to control membrane receptors, with a particular emphasis on GPCRs. The opsin-based approaches, including the ectopic expression of rhodopsin itself or a number of chimera-based methods[1-3] offer the spatial and temporal control afforded by light. In addition, they permit genetic targeting since they are expressed in cells of interest. However, it remains unclear how well these chimeric approaches mimic the precise biophysical and physiological properties of the GPCRs which they aim to probe. Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) have emerged as a very useful, general approach to control G protein activation with genetic control in a manner that is orthogonal to native pharmacology.[4,5] However, their dependence on the application of a soluble drug precludes tight temporal or spatial control and they are not likely to be powerful for probing receptor-specific properties, especially those of native receptors. Photoswitchable tethered ligands (PTLs)[6-8], including those for metabotropic glutamate receptors[8], offer genetic and spatial control and unparalleled temporal control. Importantly, since they are based on wild-type or near wild-type receptors they are well-suited to study a specific receptor, especially since the nature of photoswitchable ligand attachment permits complete receptor subtype-specificity. However, in their current incarnation they rely on overexpression and are unable to target native receptors in the absence of a transgenic animal that incorporates a labelling tag, something that is somewhat impractical for a large number of protein targets. In contrast, caged or photochromic ligands[9-11], allow for efficient targeting of native proteins with fairly high, although diffusion-limited, spatial and temporal precision. However, receptor subtype-specificity is often limited by the incomplete specificity of the parent compound that has been endowed with light sensitivity and no genetic targeting is employed, limiting the utility of these compounds for circuit and network-level analysis. The recently-reported DART approach offers an elegant approach to control native receptors *via* the attachment of a ligand to the surface of genetically-targeted cells *via* a membrane-anchored Halo-tag[12]. However, this approach does not employ any light-dependence, limiting the spatial and temporal precision, and is still

limited by the specificity of the compound that is attached to the expressed labelling tag. Finally, subtype-specific nanobodies (NBs) have been developed for GPCRs that serve as allosteric modulators to up or down-regulate receptor function.[13-15] While NBs often show enhanced subtype specificity compared to small molecule drugs, they lack spatiotemporal targeting capabilities. In principle, allosteric NBs should be capable of being genetically encoded for cell-type targeting, but this has not been demonstrated to date. In this study, we report Nanobody-Photoswitch Conjugates (NPCs) which provide the spatiotemporal control of PTLs, may be used to target native receptors, and maintain subtype specificity through combined specificity (which may be partial) of both the nanobody and the functional ligand, and can be used to target native receptors. Importantly, nanobodies may be genetically encoded and expressed in mammalian cells, allowing NPCs to potentially also operate with genetic targeting.

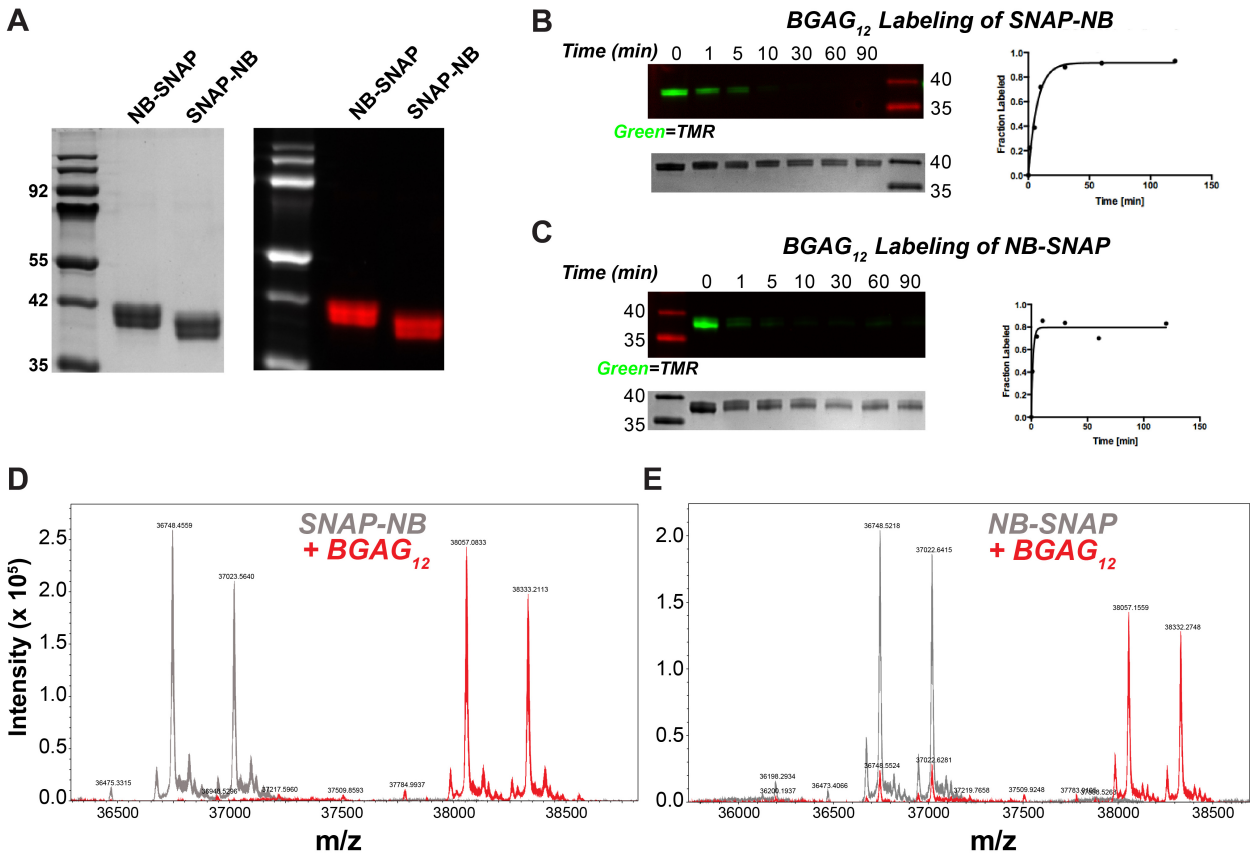

Fig. S1. Characterization of fluorophore or photoswitch labelling of SNAP-fused NBs. (A) *in vitro* labeling of SNAP in fusion with NBs with 3-fold excess of BG-TMR in 50 mM Hepes, 50 mM NaCl (pH 7.3) for 1 h. The denatured, labelled proteins were then separated by SDS-PAGE and imaged using Cy3 emission filters (605/50 nm). (B-C) SNAP fused NBs were incubated with 3-fold excess of $BGAG_{12}$ and aliquots were taken at indicated times. The aliquots were then quenched with 10-fold excess BG-TMR for 1 h, denatured and stored at -20 °C until separated by SDS-PAGE. BG-TMR was imaged with BioRad a ChemiDoc MP imager using Cy3 emission filters (605/50 nm) and analysed by ImageJ. The gels were then stained with coomassie. (D-E) Mass analysis of SNAP-NB and NB-SNAP with $BGAG_{12}$. ESI-QTOF was used to measure both labelled and unlabelled protein constructs. Two species can be observed in the spectra, one corresponds to [M-Met], the other [M-Met-2×His]. For a full summary, see Supplementary Table 2.

	Protein Mass		Protein Mass after $BGAG_{12}$ Label	
	Calculated [Da]	Observed [Da]	Calculated [Da]	Observed [Da]
SNAP-NB	37023.71	37023.56	38332.29	38333.21
	36749.43	36748.46	38058.01	38057.08
NB-SNAP	37023.71	37022.64	38332.29	38332.27
	36749.43	36748.52	38058.01	38057.16

Table S2. Mass analysis of labelled SNAP-NB and NB-SNAP. Two species can be observed in the deconvolution, one corresponding to [M-Met], the other to [M-Met-2×His].

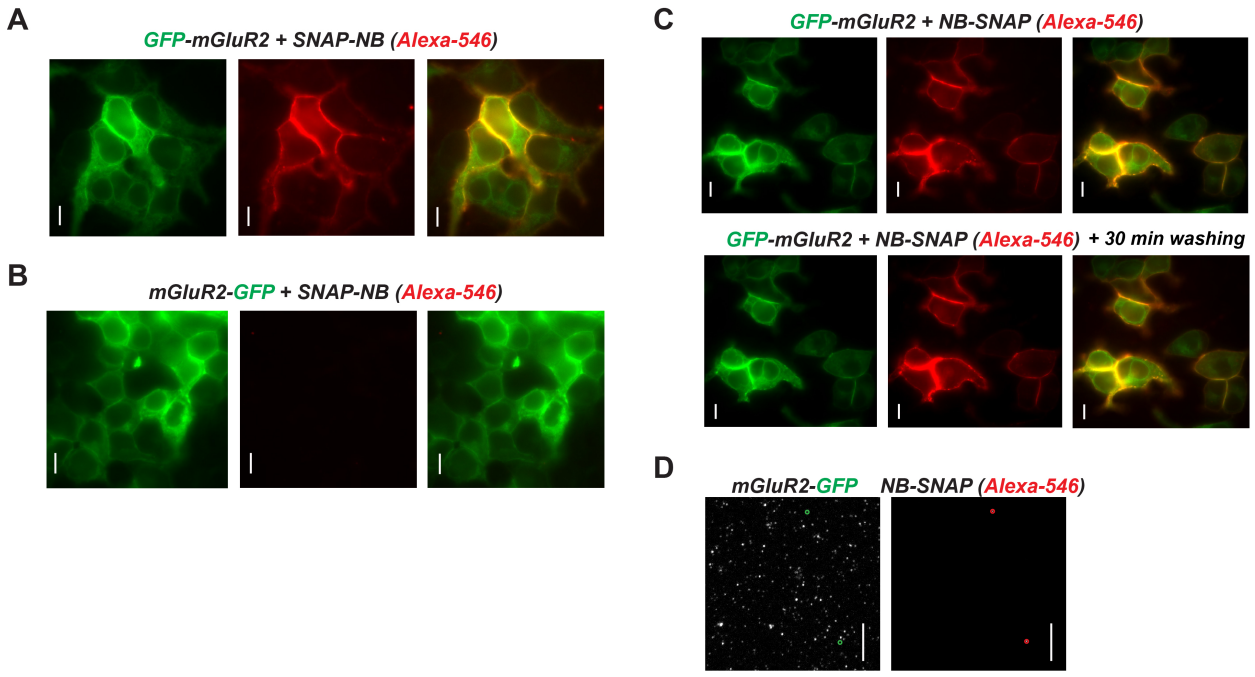

Fig. S2. Further characterization of binding properties of SNAP-tagged anti-GFP NBs with GFP-mGluR2. (A-B) Live-cell imaging of HEK293T cells transfected with GFP fusions of mGluR2 with pre-labeled Alexa-546 SNAP-NB. SNAP-tagged NBs are only able to bind the cell surface when an extracellular GFP-tag is present. Scale bar = 20 µm. (C) Alexa-546 labeled NB-SNAP on GFP-mGluR2 cannot be washed away following 30 minutes of wash at ~3 mL/min in a ~1 mL chamber. Scale bar = 20 µm. (D) Control showing minimal single molecule NB immobilization from lysate from cells expressing mGluR2-GFP, compared to GFP-mGluR2 (see Fig. 2c). Circled molecules show co-localized green (mGluR2-GFP) and red (NB-SNAP + Alexa-546) spots. Scale bars = 5 µm.

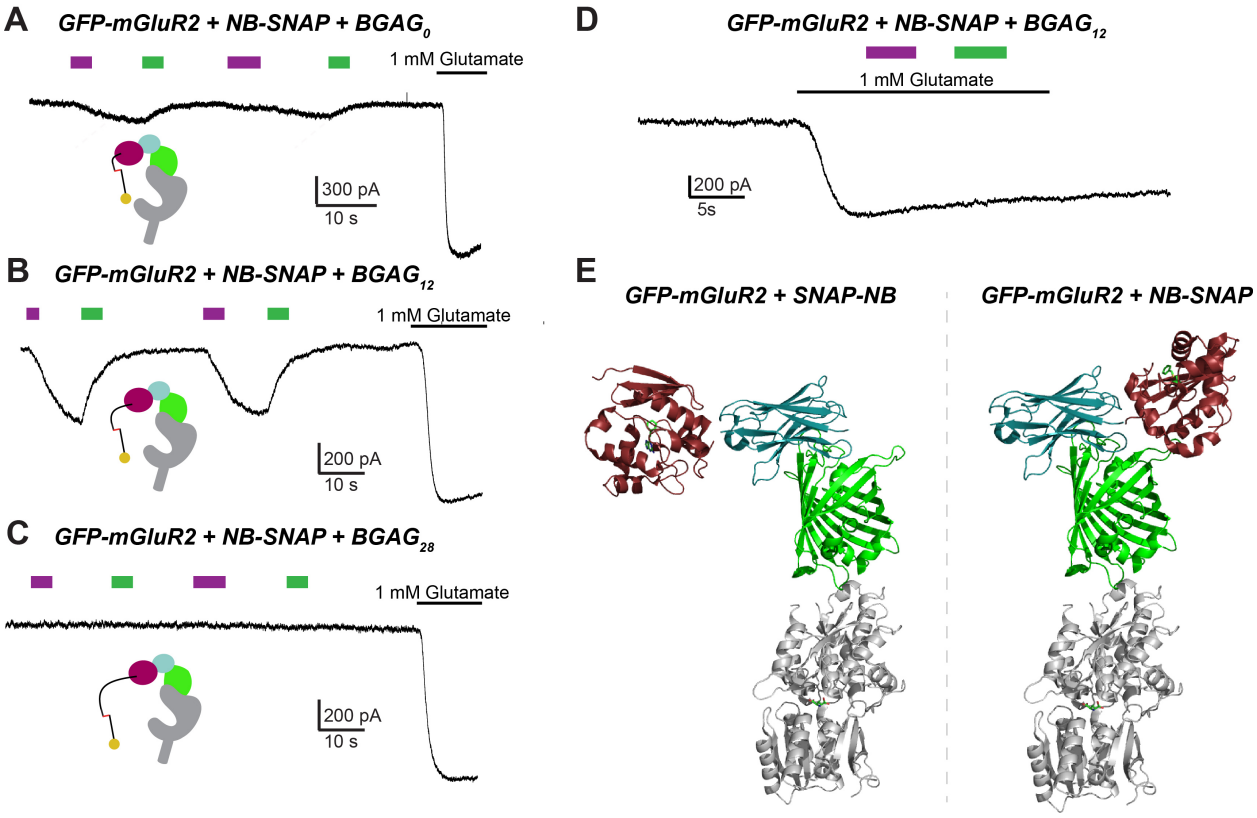

Fig. S3. Further characterization of NB-mediated photoactivation of GFP-mGluR2. (A-C) Representative traces showing relative photoswitch efficiencies for BGAG variants of different PEG linker lengths. (D) Representative trace showing that no light response is observed in the presence of saturating glutamate. (E) Alignment of crystal structures to illustrate size relationships and approximate assembly for SNAP-tagged NBs in complex with GFP-mGluR2. For this purpose, the ligand-binding domain of mGluR2 was used and placed with its *N*-terminus close to the *C*-terminus of wtGFP, which in turn was co-crystallized with the VHH nanobody. Finally, the SNAP-tag crystal structure was positioned with its *C*-terminus near the NB *N*-terminus (left) or *C*-terminus (right). It should be noted that this represents a rough model meant to merely grasp an idea of how the domains may be assembled, leaving molecular movements and alignments aside. Due to the high binding constant of VHH to wtGFP, the only flexible regions are likely located between mGluR2 and wtGFP as well as in between the SNAP tag and NB. Crystal structures used are: SNAP (magenta, pdb: 3KZZ), VHH-wtGFP (cyan and green, pdb: 3K1K)[16], ligand-binding domain of mGluR2 bound to glutamate (gray, pdb: 5CNI)[17].

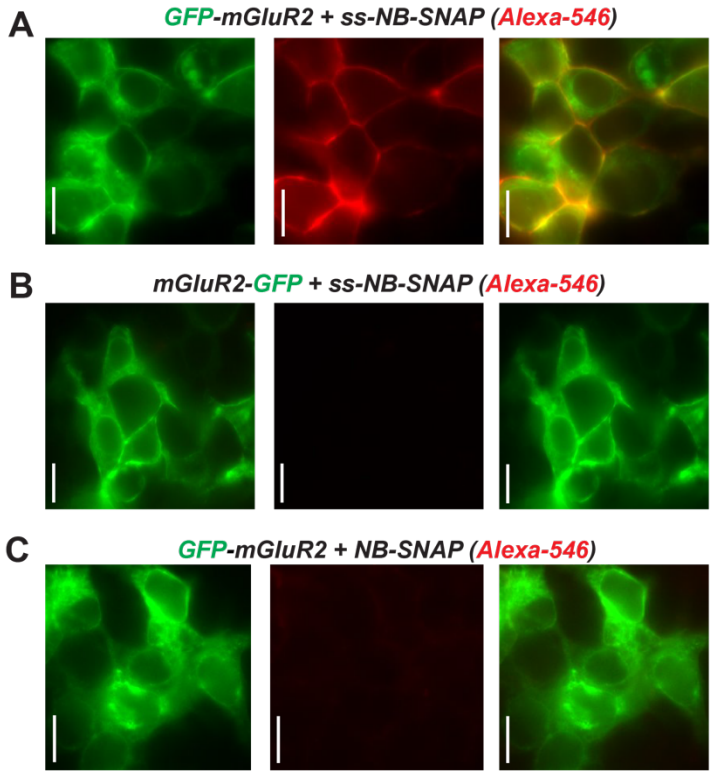

Fig. S4. Extracellular GFP targeting by NB-SNAP is dependent on the addition of an mGluR signal sequence (ss). (A-C) Live-cell imaging of HEK293T cells transfected with GFP fusions of mGluR2 along with either ss-NB-SNAP (A,B) or NB-SNAP (C). In order to immobilize NB-SNAP on the surface of the cell an extracellular GFP tag and a signal sequence is required. Scale bars = 20 μm.

Protein sequences of SNAP-NB and NB-SNAP:

SNAP-NB:

MASWSHPQFEKGADDDDKVPHMDKDCEMKRTTLDSPLGKLELSGCEQGLHEIIFLGKGTSAA
DAVEVPAPAAVLGGPEPLMQATAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLK
VVKFGEVISYSHLAALAGNPAATAAVKTALSGNPVPILIPCHRVVQGDLDVGGYEGGLAVKEWL
LAHEGHRLGKPGLGGRMAQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWYRQAPG
KEREWVAGMSSAGDRSSYEDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNVNVGFEY
WGQGTQVTVSSGAPGFSSISAHHHHHHHHHH*

NB-SNAP:

MASWSHPQFEKGADDDDKVPHMAQVQLVESGGALVQPGGSLRLSCAASGFPVNRYSMRWY
RQAPGKEREWVAGMSSAGDRSSYEDSVKGRFTISRDDARNTVYLQMNSLKPEDTAVYYCNV
NVGFEYWGQGTQVTVSSGRMDKDCEMKRTTLDSPLGKLELSGCEQGLHEIIFLGKGTSAADA
VEVPAPAAVLGGPEPLMQATAWLNAYFHQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVV
KFGEVISYSHLAALAGNPAATAAVKTALSGNPVPILIPCHRVVQGDLDVGGYEGGLAVKEWLLA
HEGHRLGKPGLGGAPGFSSISAHHHHHHHHHH*

Strep-Tag II, Enterokinase-site, SNAP, Nanobody-Enhancer, 2 amino acid linker, His-tag

Supplementary References

1. Airan, R. D., Thompson, K. R., Fenno, L. E., Bernstein, H., and Deisseroth, K. (2009) Temporally precise in vivo control of intracellular signalling, *Nature 458*, 1025-1029.
2. Li, X., Gutierrez, D. V., Hanson, M. G., Han, J., Mark, M. D., Chiel, H., Hegemann, P., Landmesser, L. T., and Herlitze, S. (2005) Fast noninvasive activation and inhibition of neural and network activity by vertebrate rhodopsin and green algae channelrhodopsin, *Proc. Natl. Acad. Sci. U. S. A. 102*, 17816-17821.
3. Oh, E., Maejima, T., Liu, C., Deneris, E., and Herlitze, S. (2010) Substitution of 5-HT1A receptor signaling by a light-activated G protein-coupled receptor, *J. Biol. Chem. 285*, 30825-30836.
4. Alexander, G. M., Rogan, S. C., Abbas, A. I., Armbruster, B. N., Pei, Y., Allen, J. A., Nonneman, R. J., Hartmann, J., Moy, S. S., Nicolelis, M. A., McNamara, J. O., and Roth, B. L. (2009) Remote control of neuronal activity in transgenic mice expressing evolved G protein-coupled receptors, *Neuron 63*, 27-39.
5. Vardy, E., Robinson, J. E., Li, C., Olsen, R. H. J., DiBerto, J. F., Giguere, P. M., Sassano, F. M., Huang, X. P., Zhu, H., Urban, D. J., White, K. L., Rittiner, J. E., Crowley, N. A., Pleil, K. E., Mazzone, C. M., Mosier, P. D., Song, J., Kash, T. L., Malanga, C. J., Krashes, M. J., and Roth, B. L. (2015) A New DREADD Facilitates the Multiplexed Chemogenetic Interrogation of Behavior, *Neuron 86*, 936-946.
6. Broichhagen, J., Damijonaitis, A., Levitz, J., Sokol, K. R., Leippe, P., Konrad, D., Isacoff, E. Y., and Trauner, D. (2015) Orthogonal Optical Control of a G Protein-Coupled Receptor with a SNAP-Tethered Photochromic Ligand, *ACS Cent. Sci. 1*, 383-393.
7. Levitz, J., Broichhagen, J., Leippe, P., Konrad, D., Trauner, D., and Isacoff, E. Y. (2017) Dual optical control and mechanistic insights into photoswitchable group II and III metabotropic glutamate receptors, *Proc. Natl. Acad. Sci. U. S. A. 114*, E3546-E3554.
8. Levitz, J., Pantoja, C., Gaub, B., Janovjak, H., Reiner, A., Hoagland, A., Schoppik, D., Kane, B., Stawski, P., Schier, A. F., Trauner, D., and Isacoff, E. Y. (2013) Optical control of metabotropic glutamate receptors, *Nat. Neurosci. 16*, 507-516.
9. Broichhagen, J., Frank, J. A., and Trauner, D. (2015) A Roadmap to Success in Photopharmacology, *Acc. Chem. Res. 48*, 1947-1960.
10. Lerch, M. M., Hansen, M. J., van Dam, G. M., Szymanski, W., and Feringa, B. L. (2016) Emerging Targets in Photopharmacology, *Angew. Chem. Int. Ed. Engl. 55*, 10978-10999.
11. Pittolo, S., Gomez-Santacana, X., Eckelt, K., Rovira, X., Dalton, J., Goudet, C., Pin, J. P., Llobet, A., Giraldo, J., Llebaria, A., and Gorostiza, P. (2014) An allosteric modulator to control endogenous G protein-coupled receptors with light, *Nat. Chem. Biol. 10*, 813-815.
12. Shields, B. C., Kahuno, E., Kim, C., Apostolides, P. F., Brown, J., Lindo, S., Mensh, B. D., Dudman, J. T., Lavis, L. D., and Tadross, M. R. (2017) Deconstructing behavioral neuropharmacology with cellular specificity, *Science 356*.
13. Jahnichen, S., Blanchetot, C., Maussang, D., Gonzalez-Pajuelo, M., Chow, K. Y., Bosch, L., De Vrieze, S., Serruys, B., Ulrichts, H., Vandevelde, W., Saunders, M., De Haard, H. J., Schols, D., Leurs, R., Vanlandschoot, P., Verrips, T., and Smit, M. J. (2010) CXCR4 nanobodies (VHH-based single variable domains) potently inhibit chemotaxis and HIV-1 replication and mobilize stem cells, *Proc. Natl. Acad. Sci. U. S. A. 107*, 20565-20570.

14. Staus, D. P., Strachan, R. T., Manglik, A., Pani, B., Kahsai, A. W., Kim, T. H., Wingler, L. M., Ahn, S., Chatterjee, A., Masoudi, A., Kruse, A. C., Pardon, E., Steyaert, J., Weis, W. I., Prosser, R. S., Kobilka, B. K., Costa, T., and Lefkowitz, R. J. (2016) Allosteric nanobodies reveal the dynamic range and diverse mechanisms of G-protein-coupled receptor activation, *Nature 535*, 448-452.
15. Scholler, P., Nevoltris, D., de Bundel, D., Bossi, S., Moreno-Delgado, D., Rovira, X., Moller, T. C., El Moustaine, D., Mathieu, M., Blanc, E., McLean, H., Dupuis, E., Mathis, G., Trinquet, E., Daniel, H., Valjent, E., Baty, D., Chames, P., Rondard, P., and Pin, J. P. (2017) Allosteric nanobodies uncover a role of hippocampal mGlu2 receptor homodimers in contextual fear consolidation, *Nat. Commun. 8*, 1967.
16. Kirchhofer, A., Helma, J., Schmidthals, K., Frauer, C., Cui, S., Karcher, A., Pellis, M., Muyldermans, S., Casas-Delucchi, C. S., Cardoso, M. C., Leonhardt, H., Hopfner, K. P., and Rothbauer, U. (2010) Modulation of protein properties in living cells using nanobodies, *Nat. Struct. Mol. Biol. 17*, 133-138.
17. Monn, J. A., Prieto, L., Taboada, L., Hao, J., Reinhard, M. R., Henry, S. S., Beadle, C. D., Walton, L., Man, T., Rudyk, H., Clark, B., Tupper, D., Baker, S. R., Lamas, C., Montero, C., Marcos, A., Blanco, J., Bures, M., Clawson, D. K., Atwell, S., Lu, F., Wang, J., Russell, M., Heinz, B. A., Wang, X., Carter, J. H., Getman, B. G., Catlow, J. T., Swanson, S., Johnson, B. G., Shaw, D. B., and McKinzie, D. L. (2015) Synthesis and Pharmacological Characterization of C4-(Thiotriazolyl)-substituted-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylates. Identification of (1R,2S,4R,5R,6R)-2-Amino-4-(1H-1,2,4-triazol-3-ylsulfanyl)bicyclo[3.1.0]hexane-2, 6-dicarboxylic Acid (LY2812223), a Highly Potent, Functionally Selective mGlu2 Receptor Agonist, *J. Med. Chem. 58*, 7526-7548.